International Journal of Agricultural Science and Research (IJASR)
ISSN(P): 2250-0057; ISSN(E): 2321-0087
Vol. 7, Issue 4, Aug 2017, 713-718

TRANS STELLAR
•Journal Publications •Research Consultancy

APPLICATION OF PUP1 MARKERS FOR IDENTIFICATION OF RICE (*ORYZA SATIVA* L.) CULTIVARS WITH TOLERANCE TO PHOSPHORUS DEFICIENCY

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ABSTRACT

Phosphorus deficiency (PD) often exists in soils that severely limit rice growth and production due to fixation in soils with high free ferric oxides and aluminum in the clay fraction. It is a widespread problem and limits access of plants to Phosphorus even if it is present in the soil. P-efficient varieties should play a major role in increasing rice yield. Therefore in this study, a set of 120 rice genotypes (composed of upland cultivars and landraces) were grown in low P soils with three different P fertilizer dose inputs (P_0, P_{25} and P_{50} $kgha^{-1}$ P) to compare P uptake efficiency. Results showed that, among the 120 genotypes, fifteen genotypes (12.5%) which produced high grain yield were further screened for probable presence of Pup1 gene by PCR amplification by two Pup1 associated insertion and deletion (InDel) markers viz, Pup1-K46 and Pup1-K52. Among the fifteen genotypes PCR screening confirmed the presence of Pup1 gene in more than 90% of the genotypes. In summary, the identified genotypes may serve as sources of PD tolerance that would be useful in future rice breeding programmes.

KEYWORDS: Rice, Genotypic Variation, Phosphorus Deficiency (PD) & Marker Assisted Selection (MAS)

Received: Jul 06, 2017; **Accepted:** Jul 31, 2017; **Published:** Aug 12, 2017; **Paper Id.:** IJASRAUG201794

Original Article

INTRODUCTION

Rice *(Oryza sativa L.)* is the most important staple food for almost more than half of the world population and is grown on over 150 million ha worldwide. Rice provides a source of carbohydrate and it provides 21% of energy and 15% of the protein requirements of human beings. Although rice farming is the major livelihood for many people world over, the global population is increasing tremendously and the targeted food production need to be increased before 2025 (Pennisi, 2008). Unsurprisingly, a large proportion of the two-thirds of the world's population that lack one or more essential mineral element in their diets rely on rice as their main staple. Phosphorus (P) is the most problematic nutrient in rice growing soils (Shen *et al*, 2011). In fact most of the rice growing soils lack the optimum levels of P for growth and development of rice (Fairhurst *et al*, 1999). Because of these reasons farmers tend to apply more P fertilizer, but consequently they pollute the environment and increase the cost of production. Governments of the developing countries including India spend lot of money on fertilizer imports. The situation is further aggravated when government funded fertilizer subsidies are placed due to numerous socio-economic and political reasons (Cordell *et al*, 2009). Organic rice growers also face difficulties as the popular organic manure such as crop stubbles and straw lack sufficient levels of P (Sirisena and Wanninayake 2014). Therefore, an alternative strategy to overcome the P fertilizer led crisis is required. To solve this problem, the plant research community considers breeding of P deficiency (PD) tolerant rice varieties as the most promising solution (Rose *et al*, 2011).

Recently quantitative genetic studies were begun to dissect the underlying genetics of PD tolerance in rice (Chin *et al*, 2011). A major quantitative trait locus (QTL) controlling PD tolerance was identified on the rice chromosome 12 using recombinant inbred lines (RILs) generated from a cross between a PD tolerant landrace *Kasalath* and sensitive landrace *Nipponbare* (Wissuwa *et al*, 2002). Later this QTL was fine mapped and labeled as *Pup1* (Chin *et al*, 2011). Markers associated to these QTL were points to the potential of identifying the lines PD tolerance information from same or different backgrounds. Moreover, this marker assisted selection (MAS) is rapidly increase the selection authenticity and efficiency PD tolerance lines.

There is growing recognition that improvements in internal P utilization efficiency are needed to complement enhanced P uptake traits if breeding P efficient crop cultivars has to be successful (Wang *et al*, 2010). Hence screening rice genotypes having good phosphorus use efficiency have to be explored and thus can be utilized in crop improvement programmes to improve yield and enhance food security in rice dependant countries. Therefore, the present study was conducted to characterize a set of local genotypes and landraces of rice cultivars evaluated for PD tolerance in field condition for phosphorus use efficiency using Pup1-K46 and Pup1-K52 marker locus to lay a foundation for MAB to produce better performing rice varieties under low P conditions.

MATERIALS AND METHODS

Plant Materials

A set of 120 rice cultivars (composed of upland cultivars and landraces) were obtained from Department of Plant Breeding and Genetics, Tamil Nadu Agricultural University (TNAU), Coimbatore, India which includes six genotypes of NILs (Near Isogenic Lines) developed from crossing the P-deficient tolerant traditional aus-type variety 'Kasalath' and the intolerant modern variety IR 74 and the other three genotypes were NILs developed by crossing Kasalath and the intolerant modern variety IR 64. These nine lines carried a chromosomal segment containing the putative quantitative trait locus (QTL) that had been transferred from the donor variety Kasalath, which acts as a positive control.

Methods

The field experiment with 120 genotypes was conducted in irrigated lowland with low phosphorus content at Agricultural Research Station, Bhavanisagar during 2014-15. Graded levels of P were added by adding single superphosphate at rates of 0, 25 and 50 kg P_2O_5 ha^{-1}. The genotypes were sown in nursery and after 23 DAS (days after sowing), the seedlings were transplanted in 1m rows with 9 plants per row. The fertilizer was applied in three splits, basal, 15 DAT (days after transplanting) and 45 DAT. Phosphorus was applied in one dose as basal application to support early growth stage of the crop. Plants were grown under wetland conditions throughout the growth period. Available phosphorus content was determined by soil analysis at the time of planting. Composite soil samples were taken from 0-20 cm depth and analyzed for Bray P (pH 5.8), which was found to be 9.2 kg ha^{-1}. The soil around the roots of each plant was loosened to a depth of 20-30 cm with a spade and the roots were slowly pulled out, which allowed most of the roots to be recovered from top soil. Roots were then washed and root length was measured. Roots, shoots and seeds were dried and weighed separately. The P content in roots, shoots and grains was determined calorimetrically by Vanadomolybdate yellow colour method (Piper, 1966).

DATA COLLECTION

After harvest, biometric traits observations *viz*, plant height (cm), root length (cm), number of tillers, 1000 grain

weight (g), grain yield (kg), primary panicle length (cm), primary panicle weight (g) and biomass at vegetative and harvest stage were measured to evaluate the phosphorus use efficiency (PUE) of the genotypes under different treatments.

DNA EXTRACTION AND PCR AMPLIFICATION

Fresh rice leaves were collected from 20 days old seedlings and frozen in liquid nitrogen immediately, and stored at −20°C. Total genomic DNA was isolated using a modified cetyltrimethyl ammonium bromide (CTAB) method (Doyle, 1990). The quality and quantity of DNA were checked by agarose gel electrophoresis. The final concentration of all the samples was adjusted to 25 ng μL^{-1}. The DNA templates from all the fifteen rice genotypes accessions were amplified using the *Pup1* gene-specific primers namely Pup1-K46 and Pup1-K52 (Table.1). PCR amplification was performed in a Thermal Cycler (Eppendorf, Germany). A volume of 15 µL of PCR mix contained 1.5 µL of 10X assay buffer, 0.5 µL of 2.5 Mm dNTPs, 0.20 µL of 0.5 unit μL^{-1} of *Taq* polymerase 1.0 µL of 10 µM primer and 3.0 µL of 25 ng μL^{-1} DNA. The temperature cycles were as follows: 4 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 57°C, 1 min extension at 72°C. The final extension step was extended to 5 min at 72°C and finally maintained at 4°C. The amplified products were separated on a 3% agarose gel.

DATA ANALYSIS

The field collected data were subjected to single-environment ANOVAs to evaluate differences in yield characters and P concentration in grain and straw among genotypes within each environment and to calculate least square means for these variables in each environment using SAS software (SAS Institute Inc, 2012). The phosphorus use efficiency (PUE) of the rice genotypes grown under field condition was calculated as suggested by Syers et al. (2008).

RESULTS AND DISCUSSIONS

Genotypic Variation for P Use Efficiency

In case of screening of 120 genotypes, the range for each character under each individual treatment was given in Table 2. In case of plant height, IR 50 performed well under minus-P condition. CO 43 exhibited shorter stature under the treatment 25 kg ha^{-1} of P and IR 74-Pup1-B had shorter height when 50 kg ha^{-1} of P was applied. For the character number of tillers per plant, the variety Bharathi performed best at minus P conditions and Cult 1177 performed better when P was applied. CO 10 had significant total dry straw yield under minus-P condition. Cult 1177 had high dry straw yield under 25 kg ha^{-1} of P applied condition, whereas ADT 44 performed well for total dry straw yield when 50 kg ha^{-1} of P was applied. CO 45 exhibited significant primary panicle weight under minus-P condition. ASD 16 showed good primary panicle length and GEB 24 had significant single straw weight under minus-P condition. For the main economic trait grain yield, ASD 16 outweighed all the other 119 varieties under all the three treatments. For the character root length, which is a major trait for evaluating the P use efficiency, CO 46 performed well under minus-P conditions, with a root length of 26.9 cm. The traditional landrace Kasalath and the NILs IR 74-Pup1-A, IR 64-Pup1-F, IR 74-Pup1-G, IR 74-Pup1-D, IR 74-Pup1-E and IR 64-Pup1-M with the Pup1 QTL exhibited root length of more than 20 cm which was more than that of most of the modern varieties. Another trait, root weight per plant indicates better P use efficiency of genotypes. And CO 44 and the NILs IR 74- Pup1-C, IR 74-Pup1-G and IR 74-Pup1-D performed well under minus P conditions.

Genotypic Screening for Pup1 Marker

Molecular markers are the popular approaches for discovering and tagging novel genes and alleles. These

technologies can be effective in breeding programs through their use in Molecular Assisted Selection (MAS). MAS for PD-tolerance related QTLs under deficient has been effective in rice (Chin et al, 2010). The stability among various genotypes to select high yielding and PD tolerance rice lines is the key criterion for breeding programs. A high level of tolerance in rice genotypes against PD has been studied (Chin et al, 2010). But, identification and evaluation of rice tolerance lines against PD aiming at to combine phenotype screening linked with gene using PCR based markers is a better strategy in rice breeding.

Based on the grain yield character, fifteen genotypes (TKM 12, RMD 1, GEB 24, PMK 2, CO 34, ASD 16, IR 20, IR 72, IR 64, ADT 39, CO 46, TKM 3, TKM 6, TKM 9 and Kasalath) which possess highest grain yield among the 120 genotypes. These fifteen genotypes were selected and screened for probable presence of *Pup1* gene, which is the major PD tolerance gene in rice. Two *Pup1* gene linked markers Pup1-K46 and Pup1-K52 were used to test the genotypes by PCR amplification. The dominant marker Pup1-K46 produced the amplicon size of 523 bp in fourteen genotypes (Figure.1) and similarly Pup1-K52 dominant marker also produced the expected amplicon size of 505 bp in all the genotypes except IR 72 (Figure. 2). The gene-based *Pup1* markers that are now available have been extensively tested and provide sufficient details on the different *Pup1* haplotypes present in the rice genotypes. These results are in accordance with the reports of Heuer et al, (2009) and Tyagi et al, (2012).

Table 1: List of Markers Used to Screen Presence of Pup1 QTL

Marker Name	Sequence	Gradient Range (0 C)	Annealing Temp (o C)	Kasalath Allele (bp)
Pup1-K46	5'-TGAGATAGCCGTCAAGATGCT-3' (F) 5'-AAGGACCACCATTCCATAGC-3' (R)	54-59	59	523
Pup1-K52	5'ACCGTTCCCAACAGATTCCAT-3' (F) 5'CCCGTAATAGCAACAACCCAA-3' (R)	54-59	59	505

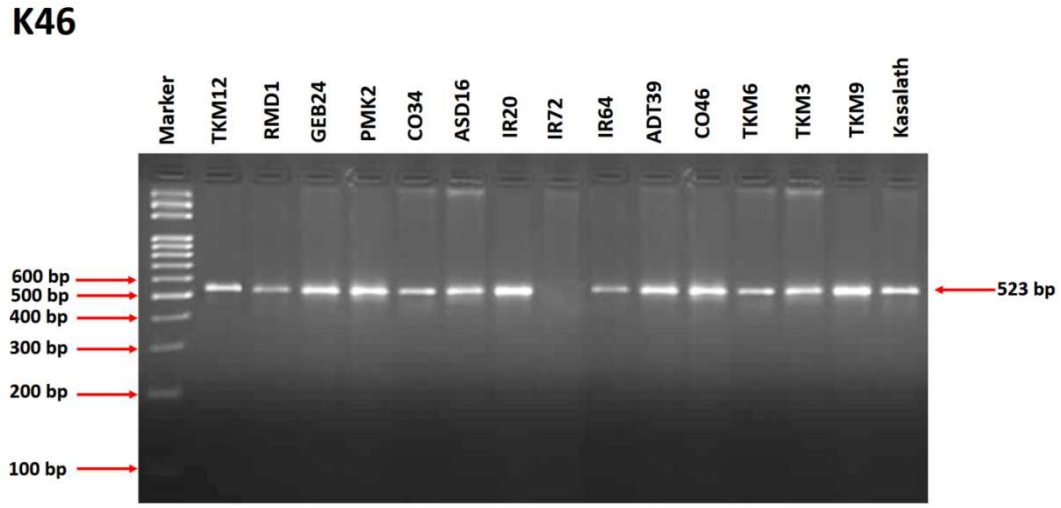

Figure 1: Amplicons Obtained with Pup1-K46

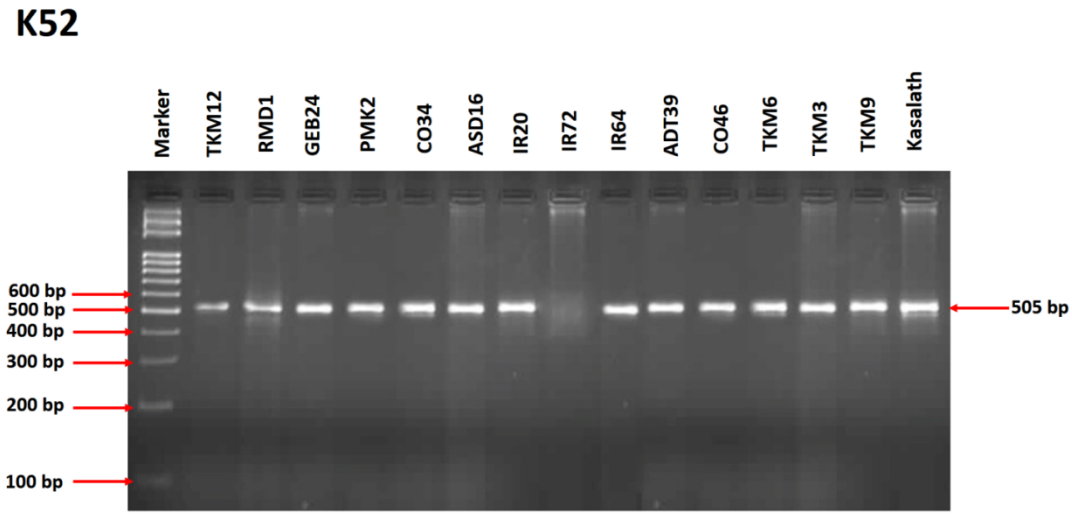

Figure 2: Amplicons Obtained with Pup1-K52

Table 2: Comparison of Grand Means of 120 Rice Genotypes Grown at Three Levels of Pat Harvest Stage

Characters	Range (kg ha^{-1})			Grand Mean (kg ha^{-1})		
	P0	P25	P50	P0	P25	P50
Plant Height (cm)	72.4 – 141.3	64.7 – 142.1	69.5 – 143.7	94.5	97.3	99.0
Root Length cm)	8.0 – 26.9	8.0 – 24.8	9.1 – 24.2	17.9	16.9	16.7
No. of tillers	16.7 – 47.7	17.3 – 40.3	17.3 – 44.0	29.9	27.3	28.6
Straw Yield (kg ha^{-1})	0.4 – 2.6	0.4 – 2.5	0.5 – 2.5	1.1	1.1	1.1
1000 Grain Weight (g)	12.2 – 32.7	13.2 – 27.9	12.9 – 32.3	21.7	21.8	22.5
Grain Yield (kg ha^{-1})	0.11 – 0.5	0.09 – 0.51	0.12 – 0.5	0.3	0.3	0.3
Primary Panicle Weight (g)	0.9 – 4.0	0.9 – 4.1	0.8 – 4.0	2.4	2.2	2.1
Primary Panicle Length (cm)	20.2 – 33.9	18.5 – 33.8	19.0 – 34.9	25.2	24.7	25.7
Straw Weight (g plant^{-1})	12.8 – 54.0	13.5 – 52.7	16.8 – 57.7	31.5	29.7	31.9
Root Weight (g plant^{-1})	13.3 – 45.3	13.0 – 47.8	11.3 – 46.8	23.3	23.3	23.3
PUE (%)		39.8 – 192.6	29.5 –108.1	107.7	103.2	105. 4

CONCLUSIONS

In this study, fourteen genotypes were identified as PD tolerant genotypes, which could provide an elite array of tolerance source for effective breeding of rice cultivars to PD. Based on PUE (%) cultivars like PMK 2, CO 46, TKM 3, GEB 24, IR 20 and TKM 6 were found to be phosphorus deficient tolerant rice genotypes. It is also confirmed that these genotypes may possess pup1 gene as evident from the PUE (%) calculated based on low phosphorus soil screening. Further studies are required to validate the presence of pup1 gene by sequencing technologies. In addition, many P-deficient soils are constrained by other stresses like aluminium toxicity, salinity, and nematodes etc that restrict root growth and interfere

with *Pup1* phenotyping. It is concluded that those genotypes may serve as phosphorus tolerant genotypes in future breeding programs and genetic studies on phosphorus deficiency tolerance to detect potentially novel genetic mechanisms.

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